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Mice genetically inactivated in interleukin-17A receptor are defective in long-term control of *Mycobacterium tuberculosis* infection

Danielle Freches,[1] Hannelie Korf,[1,*] Olivier Denis,[1] Xavier Havaux,[2] Kris Huygen[1] and Marta Romano[1]

[1]*Service Immunology, Scientific Institute of Public Health WIV-ISP (Site Ukkel), Brussels, and* [2]*Cardiovascular Research Laboratory, Medical School, Université Catholique de Louvain, Brussels, Belgium*

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* Present address: Laboratory of Experimental Medicine and Endocrinology, Katholieke Universiteit Leuven, Gasthuisberg O&N1, Leuven, Belgium.
Correspondence: Dr Kris Huygen, WIV-ISP (Site Ukkel) Service Immunology, 642 Engelandstraat, B1180 Brussels, Belgium.
E-mail: kris.huygen@wiv-isp.be
Senior author: Kris Huygen

Summary

Interleukin-17A (IL-17A), a pro-inflammatory cytokine acting on neutrophil recruitment, is known to play an important role during *Mycobacterium tuberculosis* infection, but the role of IL-17A receptor signalling in immune defence against this intracellular pathogen remains poorly documented. Here we have analysed this signalling using C57BL/6 mice genetically inactivated in the IL-17 receptor A subunit (IL-17RA$^{-/-}$). Although early after infection bacterial growth was controlled to the same extent as in wild-type mice, IL-17RA$^{-/-}$ mice were defective in exerting long-term control of *M. tuberculosis* infection, as demonstrated by a progressively increasing pulmonary bacterial burden and shortened survival time. Compared with infected wild-type mice, IL-17RA$^{-/-}$ mice showed impaired recruitment of neutrophils to the lungs at the early but not the late stage of infection. Pulmonary tumour necrosis factor-α, IL-6 and particularly IL-10 levels were decreased in the absence of IL-17RA signalling, whereas IL-1β was increased. CD4$^+$-mediated and $\gamma\delta$-mediated IL-17A production was dramatically increased in IL-17RA$^{-/-}$ mice (confirming part of their phenotype), whereas production of interferon-γ and expression of the bactericidal enzyme inducible nitric oxide synthase were not affected. Collectively, our data suggest that early but not late neutrophil recruitment is essential for IL-17A-mediated long-term control of *M. tuberculosis* infection and that a functional interferon-γ response is not sufficient to control *M. tuberculosis* growth when the IL-17RA pathway is deficient. As treatment of auto-immune diseases with anti-IL-17A antibodies is actually being tested in clinical studies, our data suggest that caution should be taken with respect to possible reactivation of tuberculosis.

Keywords: interleukin-17A; interleukin-17RA$^{-/-}$; tuberculosis.

Introduction

Tuberculosis (TB) caused by infection with *Mycobacterium tuberculosis* remains a major health problem with 8·8 million new cases and 1·45 million deaths reported in 2010. In addition, it is estimated that one-third of the world population is latently infected with *M. tuberculosis.*[1] So far, the attenuated *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) strain is the only available TB vaccine. However, BCG is poorly efficient against pulmonary TB, which is the most common form of TB in adults.[2,3] To develop more effective vaccination strategies, a better understanding of the protective immune response against *M. tuberculosis* is required. Protective immunity against *M. tuberculosis* depends on interactions between specifically sensitized T cells [CD4$^+$ T helper type 1 (Th1), CD8$^+$, CD1-restricted and $\gamma\delta$ cells) and infected macrophages. *Mycobacterium tuberculosis* stimulates the production of interleukin-12 (IL-12) by macrophages and dendritic cells (DCs), leading to the induction of a Th1 type immune response, with interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) considered to be the pivotal cytokines essential to control *M. tuberculosis* growth and granuloma formation, respectively.[4] The role

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played by other T-cell subsets such as Th2 cells, regulatory T cells, Th17 cells and follicular helper T cells in the response against *M. tuberculosis* is still not fully understood.

The cytokine IL-17A is the founding member of the IL-17 family (IL-17A to IL-17F). Interleukin-17A is a pro-inflammatory cytokine primarily produced by $\gamma\delta$ T cells and Th17 T cells that promotes granulopoiesis and the recruitment of neutrophils to the site of infection.[5] In the context of auto-immune disease models – such as experimental autoimmune encephalitis as a model for multiple sclerosis and collagen-induced arthritis as a model for rheumatoid arthritis – IL-17A has been shown to have detrimental effects by promoting inflammation.[6,7] On the other hand, IL-17A is important for protection against fungi such as *Candida albicans* or extracellular bacteria such as *Klebsiella pneumoniae*.[8] Interleukin-17A induces the production of a wide variety of molecules including cytokines (IL-6, TNF-α, IL-1β), chemokines [KC (CXCL1), LIX (CXCL5), IP-10 (CXCL10)] implicated in granulopoiesis, neutrophil or lymphocyte recruitment and inflammation, and anti-microbial molecules [inducible nitric oxide synthase (iNOS), lipocalin-2 (Lcn2)].[5,8,9]

The role of IL-17A in immune defence against *M. tuberculosis* is complex. Happel *et al.*[10] have shown that transient expression of IL-23 (which in conjunction with IL-6 and transforming growth factor-β stimulates the differentiation of Th17 cells) in the lungs of *M. tuberculosis*-infected mice increases the production of IFN-γ and IL-17A by local T cells, resulting in a significantly reduced number of bacteria in the lungs. Also, *IL-17A*$^{-/-}$ mice are defective in controlling a high intratracheal dose of *M. tuberculosis*[11] and, finally, IL-23 has been found to be essential for the IL-17A response during tuberculosis, but dispensable for protection and antigen-specific IFN-γ responses if IL-12p70 is available early during infection.[12] More recently, IL-23 was shown to be required for long-term control of *M. tuberculosis*[13], and moreover, the IL-23/IL-17A axis is important in vaccine-induced protection by promoting an optimal IFN-γ recall response in the lungs.[14] Finally, we have shown that BCG-induced protection against *M. tuberculosis* in mice treated with an auto-vaccine against IL-12p70 (which specifically neutralized IL-12 while leaving the IL-23 axis intact) was only marginally affected by the IL-12 neutralization and that the concomitant decreased IFN-γ response could be compensated by increased production of TNF-α, IL-6 and IL-17A.[15] However, strong IL-17A responses do not necessarily correlate with strong protection, as boosting BCG vaccinated mice with MVA85A did not increase the protective efficacy of the BCG vaccine against *M. tuberculosis* in long-term survival experiments, despite a dramatically increased IL-17A and IFN-γ response.[16] Repeated BCG vaccination of *M. tuberculosis*-infected mice also results in increased IL-17A responses, neutrophil recruitment and concomitant lung tissue damage.[17]

Here, we have revisited the role of IL-17A during *M. tuberculosis* infection using mice genetically inactivated in IL-17 receptor A subunit (IL-17RA$^{-/-}$ mice). The IL-17A receptor is constitutively expressed on a wide variety of tissues (lungs, spleen, liver) and on many cell types (fibroblasts, epithelial cells, DCs, macrophages, lymphocytes, granulocytes).[9] Wild-type (WT) C57BL/6 mice and IL-17RA$^{-/-}$ mice were infected with *M. tuberculosis*, bacterial replication and disease progression was followed in long-term survival experiments, changes in pulmonary cell recruitment were monitored in bronchoalveolar lavage (BAL) fluid and finally lung cytokine expression was analysed by quantitative RT-PCR, flow cytometry, ELISA and ELISPOT.

Materials and methods

Animals

Wild-type C57BL/6 and IL-17RA$^{-/-}$ mice were bred in the animal facilities of the Scientific Institute of Public Health (Brussels, Belgium). The IL-17RA$^{-/-}$ mice were previously described[18] and provided as breeding pairs by Dr B. Ryffel (CNRS, Orléans, France) and Dr A. Lemoine (IMI, ULB, Brussels, Belgium). All mice were 6–9 weeks old at the start of the experiments.

Mycobacterium tuberculosis infection

Mice were inoculated intratracheally [10^3 or 5×10^3 colony-forming units (CFU)] or intravenously (2×10^5) with *M. tuberculosis* H37Rv, grown for 2 weeks as a surface pellicle on Sauton medium and stored frozen in aliquots at $-80°$.[19,20] Lung and spleen homogenates were plated on 7H11 Middlebrook agar supplemented with 10% oleic acid-albumin-dextrose-catalase and bacterial colonies were enumerated visually after 3 weeks. Animals were killed by cervical dislocation at various indicated time-points after infection. Groups of six to ten mice were monitored weekly for mortality and cachexia. Mice were killed when body weight dropped below 80% of the initial body weight. All animal experiments were performed according to rules established by the ethics committee of the Scientific Institute of Public Health (WIV-ISP)/Veterinary and Agrochemical Research Centre (CODA-CERVA) (permit 060202-02).

Histological analysis

Lungs were excised and fixed in 4% buffer formaldehyde. After fixation, the lungs were classically processed for paraffin embedding. Tissues were sliced in 5-μm thick sections and further stained with haematoxylin & eosin according

to standard histological procedure. Acid-fast staining of the mycobacteria was performed according to the manufacturer's instructions (AFB staining kit; Thermo Shandon, Runcorn, UK) and counterstained with malachite green.

Bronchoalveolar lavage (BAL)

Mice were killed, the trachea was cannulated and BAL was performed three times with 1 ml ice-cold Hanks' balanced salt solution (Invitrogen, Carlsbad, CA) supplemented with 0·05 mM EDTA. The cells of the three lavages were pooled per mouse to analyse the composition of cells infiltrating the lungs.

Assessment of leucocyte distribution in BAL fluid

The total volume and cell number recovered from the BAL were recorded. Number and composition of cells infiltrating the lungs were determined using cytospin for individual mice. Cells were classified as macrophage/DC, neutrophil and lymphocyte using standard morphological criteria after staining with Kwikdiff coloration (Thermo-Scientific, Waltham, MA). At least 200 cells were counted per cytospin preparation, and the absolute number of cells for each cell type was calculated.

Intracellular IFN-γ or IL-17A cytokine staining

The lungs from five mice per group were removed aseptically and homogenized by gentle disruption in a Dounce homogenizer. Pooled lung cells were cultured in complete medium: RPMI-1640 (Invitrogen) supplemented with 10% fetal calf serum (Greiner Bio-One, Monroe, NC), 5×10^{-5} M 2-mercaptoethanol, penicillin, streptomycin, amphotericin B (Fungizone; Invitrogen) and stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) (Sigma, St Louis, MO) for 1 day at 37° and 5% CO_2. Cells were incubated for 4 hr in the presence of GolgiPlug (BD Biosciences, San Jose, CA). The cells were washed in staining buffer (PBS + 0·1% NaN_3 + 5% fetal calf serum) and then treated with FcγRIII/II (Fc blocker; BD Pharmingen, Franklin Lakes, NJ). For surface staining, biotin-conjugated anti-CD4, CD8a and γδ monoclonal antibody (BD Pharmingen) with streptavidin-conjugated Alexa Fluor 488 (Invitrogen) or CD4-FITC, CD8a-FITC, γδ-phycoerythrin (PE), CD19-PE, CD49/Pan-NK-PE (BD Pharmingen) were used. Intracellular cytokine staining was performed on surface-stained cells using PE-conjugated anti-IFN-γ or anti-IL-17A monoclonal antibody (BD Pharmingen) after permeabilization of the cells with Cytofix/Cytoperm kit (BD Biosciences). Fluorescent isotype control antibodies were used as negative controls and no unspecific staining was observed (data not shown). Fluorescence was analysed in a FACSCalibur flow cytometer using Cell-Quest software (BD Biosciences).

In vitro cytokine production

At 5 and 12 weeks post-infection, the lungs from five mice per group were removed aseptically, homogenized by gentle disruption in a Dounce homogenizer and pooled. Leucocytes were left unstimulated or were stimulated with immunodominant, I-A^b-restricted peptide ESAT-$6_{(1–20)}$ (10 μg/ml) or polyclonal mitogen concanavalin A (Con A; 4 μg/ml) and incubated at 37° in round-bottom, 96-well microplates in a humidified CO_2 incubator. After 72 hr, supernatants were stored at − 20° and used for ELISA or flow cytometric cytokine assays.

IFN-γ and IL-17A ELISA

Interferon-γ and IL-17A were quantified in 72-hr culture supernatants of lung cultures using a sandwich ELISA as described previously.[15,16]

IL-17A ELISPOT

The number of IL-17A-producing cells was quantified by ELISPOT as described previously using biotinylated rat anti-mouse IL-17A antibody (BD Pharmingen) and ExtrAvidin®-Alkaline Phosphatase (Sigma).[21]

Flow cytometric cytokine assays

Pulmonary cell supernatants from three wells were pooled after 72 hr of culture and assayed simultaneously by flow cytometry for the presence of IL-6, IL-1β, KC (CXCL1), IP-10 (CXCL10), IL-10, TNF-α and IL-13 using the mouse FlowCytomix Multiple Analyte Detection kit (eBioscience, San Diego, CA). Results were analysed in a FACSCalibur using the FlowCytomix Pro 2.4 software (BD Biosciences).

Expression profiling of lung cells by quantitative RT-PCR

Total RNA was extracted from lung cells using TRI Reagent according to the manufacturer's instructions (Sigma). Complementary DNA was synthesized using the SuperScript II RNase H^- Reverse Transcriptase from Invitrogen. The forward and reverse primers used are shown in Table 1. Hydroxymethylbilane synthase (HMBS) mRNA was used as the reference housekeeping gene for normalization. The level of target mRNA, relative to the mean of the reference housekeeping gene, was calculated as described previously.[22]

Statistics

Values were expressed as mean ± SD unless stated otherwise. Survival data were analysed using the method of Kaplan–Meier and Logrank test using prism version 4.0

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Table 1. Primers used for quantitative RT-PCR profiling

Primer	Sequence
LIX (CXCL5) forward	5′-GGTCCACAGTGCCCTACG-3′
LIX (CXCL5) reverse	5′-GCGAGTGCATTCCGCTTA-3′
Lcn2 forward	5′-ACATTTGTTCCAAGCTCCAGGGC-3′
Lcn2 reverse	5′-CATGGCGAACTGGTTGTAGTCCG-3′
iNOS forward	5′-GGAGCAGGTGGAAGACTATTTCTT-3′
iNOS reverse	5′-CATGATAACGTTTCTGGCTCTTGA-3′
LRG47 forward	5′-AGCCGCGAAGACAATAACTG-3′
LRG47 reverse	5′-CATTTCCGATAAGGCTTGG-3′
Tbet forward	5′-CCTACCAGAACGCAGAGATCACT-3′
Tbet reverse	5′-CACTCGTATCAACAGATGCGTACAT-3′
RORγt forward	5′-CCCCCTGCCCAGAAACACT-3′
RORγt reverse	5′-GGTAGCCCAGGACAGCACAC-3′
Foxp3 forward	5′-GGCCCTTCTCCAGGACAGA-3′
Foxp3 reverse	5′-GCTGATCATGGCTGGGTTGT-3′
GATA3 forward	5′-GGCAGAAAGCAAAATGTTTGCT-3′
GATA3 reverse	5′-TGAGTCTGAATGGCTTATTCACAAT-3′
HMBS forward	5′-GAAACTCTGCTTCGCTGCATT-3′
HMBS reverse	5′-TGCCCATCTTTCATCACTGTATG-3′

HMBS, hydroxymethylbilane synthase; iNOS, inducible nitric oxide synthase.

(GraphPad, La Jolla, CA). The other data were analysed using Student's *t*-test.

Results

IL-17RA-deficient C57BL/6 mice are incapable of exerting long-term control of *M. tuberculosis* infection

To determine the importance of IL-17RA signalling in protective immunity against *M. tuberculosis*, C57BL/6 mice genetically inactivated in IL-17RA[18] and WT mice were infected intratracheally with 10^3 CFU of *M. tuberculosis* H37Rv and monitored for bacterial replication in spleen and lungs at different time-points post-infection. The bacterial burden in lungs of IL-17RA$^{-/-}$ mice and control WT mice increased 100-fold between week 1 and week 5, and was comparable in both groups during this first stage. These bacterial numbers were maintained after 12 and 20 weeks of infection in WT mice. However, in IL-17RA$^{-/-}$ mice bacterial numbers had increased another 10-fold at 12 weeks post-infection and 70-fold at 20 weeks post-infection (Fig. 1a). Likewise, bacterial load in spleens of IL-17RA$^{-/-}$ mice and control WT mice was comparable at 5 weeks post-infection, but after 12 weeks of infection, the bacterial dissemination to the spleen was slightly (Δ Log$_{10}$ 0·4), but significantly higher in IL-17RA$^{-/-}$ than in WT mice (Fig. 1b).

Wild-type C57BL/6 mice are among the most resistant mouse strains with respect to *M. tuberculosis* and they will survive for more than 30 weeks following aerosol or intravenous infection.[23] Up to 14 weeks post-infection, WT and IL-17RA$^{-/-}$ mice did not differ in their survival curves, although WT mice, but not IL-17RA$^{-/-}$, mice gradually gained weight. However, IL-17RA$^{-/-}$ mice progressively lost control of the infection, resulting in a gradual weight loss and accelerated mortality (Fig. 1c,d). The IL-17RA$^{-/-}$ mice had a median survival time of 18 weeks after infection, whereas median survival time for control WT mice was 35 weeks ($P < 0{\cdot}005$). At 12 and 20 weeks post-infection, histological analysis of the lungs revealed no gross difference in inflammation between IL-17RA$^{-/-}$ mice and WT mice (Fig. 1e). However, (and confirming the CFU results) acid-fast bacilli were more readily detected in lungs from IL-17RA$^{-/-}$ mice than in lungs from WT mice after 20 weeks of infection (Fig 1e). These results demonstrate that the IL-17RA signalling pathway is required for the long-term control of *M. tuberculosis* bacterial growth in the lungs but apparently without a major effect on pulmonary tissue inflammation. This increased susceptibility of IL-17RA$^{-/-}$ mice was also observed in long-term survival experiments after intratracheal infection with a fivefold higher dose of 5×10^3 CFU (Supplementary material, Fig. S1A) and after intravenous infection with 2×10^5 CFU (Supplementary material, Fig. S1B).

Impaired cell recruitment to the lungs of IL-17RA$^{-/-}$ mice after *M. tuberculosis* infection

The principal role of IL-17A is to promote granulopoiesis and recruitment of neutrophils to the lungs. Moreover, IL-17A promotes recruitment of CD4$^+$ lymphocytes in a context of recall response after *M. tuberculosis* infection.[14] We therefore analysed the composition and the number of cells infiltrating the lungs after infection by analysing morphologically the cells isolated by BAL (Fig. 2). At 2 weeks after infection, BAL fluid of WT mice was composed of neutrophils and macrophages/DC. Lymphocytes were not detected at this time. No neutrophils were found in the BAL fluid of IL-17RA$^{-/-}$ mice at 2 weeks post-infection and the number of macrophages was also very low and comparable with that of naive mice in three of five animals. At 5 weeks after infection, a higher number of macrophages/DCs, lymphocytes and neutrophils were detected in BAL fluid of WT mice, whereas BAL fluid of infected IL-17RA$^{-/-}$ mice still contained significantly fewer macrophages/DCs and lymphocytes and a minimal neutrophil number. At 12 weeks post-infection, the number of neutrophils was similar in BAL fluid of IL-17RA$^{-/-}$ mice and of WT mice. Macrophage/DC content was also comparable between the two groups at this time-point, whereas lymphocyte content remained significantly lower in the BAL fluid of IL-17RA$^{-/-}$ mice. These results demonstrate a role of the IL-17RA signalling pathway in the early recruitment of neutrophils and macrophages/DCs into the lungs after intratracheal

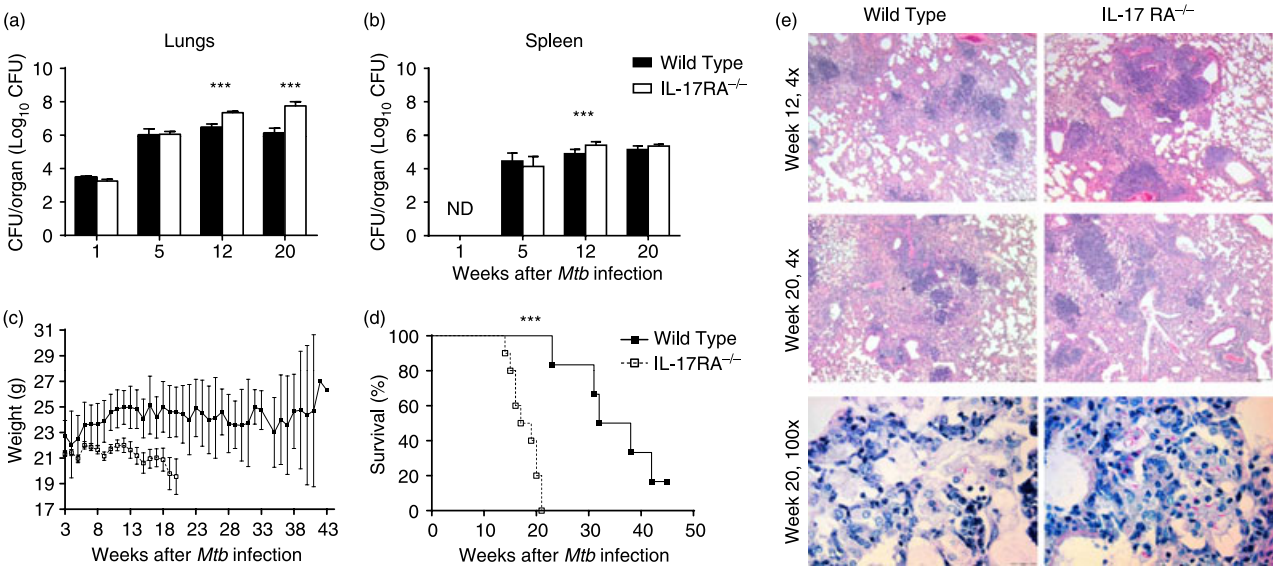

Figure 1. Wild-type (WT) C57BL/6 (■) and interleukin-17 receptor subunit A-deficient (IL-17RA$^{-/-}$) mice (□) were infected intratracheally with 10^3 colony-forming units (CFU) of *Mycobacterium tuberculosis*. (a, b) Bacterial replication (Log_{10} CFU) in the lungs and spleen of WT C57BL/6 (n = 5) and IL-17RA$^{-/-}$ (n = 5) mice was measured at different time-points after infection. (c) Mean body weight and (d) percentage of surviving mice was monitored weekly [(■, n = 6), (□, n = 10)]. (e) Histological analysis of haematoxylin & eosin-stained lung sections at 12 and 20 weeks after infection (original magnification 4×). Acid fast staining of the sections showed the presence of mycobacteria as pink rods (original magnification 100×). Bars represent the mean values obtained for individually tested animals ± SD. Statistical significance between groups is represented in the figure by ***(P < 0·005) and was evaluated by Logrank test (survival) or Student's t-test (bacterial load). ND = not done.

M. tuberculosis infection and in the recruitment of lymphocytes at the later stage of infection.

Increased IFN-γ and IL-17A production in lungs of IL-17RA$^{-/-}$ mice

To determine the phenotype of immune cells in the lungs of *M. tuberculosis*-infected mice, FACS analysis was performed on total pulmonary cells 5 weeks after infection. The percentage of CD4$^+$ T cells, CD8$^+$ T cells and CD19$^+$ B cells was comparable in WT and IL-17RA$^{-/-}$ mice, whereas percentages of $\gamma\delta$ T cells and CD49/Pan-NK cells were about twofold higher in IL-17RA$^{-/-}$ mice (Fig. 3a and Supplementary material, Figure S2). Intracellular cytokine staining following stimulation with PMA + Ionomycin showed comparable IFN-γ responses in WT and

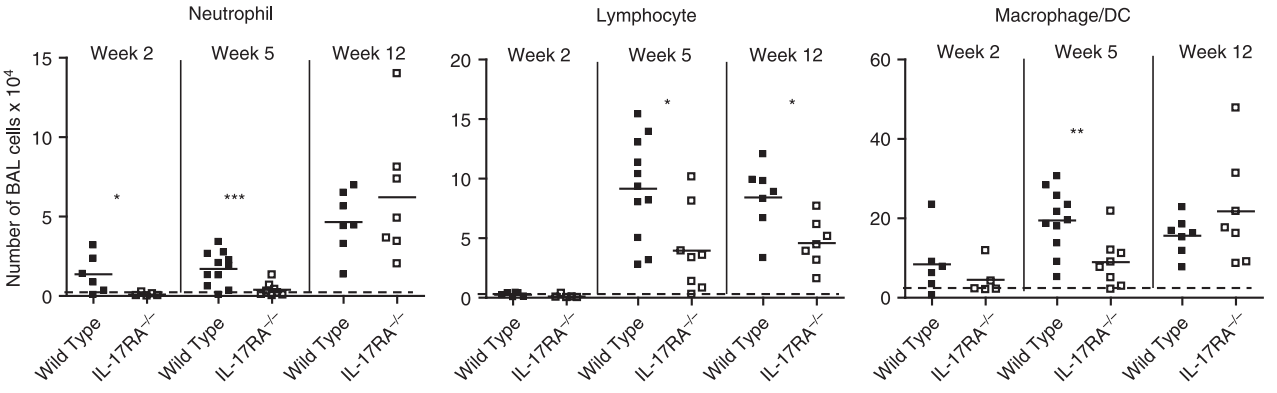

Figure 2. Differential cell infiltration in the bronchoalveolar lavage (BAL) of wild-type (WT) C57BL/6 (■) and interleukin-17 receptor subunit A-deficient (IL-17RA$^{-/-}$) (□) mice at 2, 5 and 12 weeks after intratracheal instillation of *Mycobacterium tuberculosis* (10^3 colony-forming units). Absolute numbers of neutrophils, lymphocytes and macrophages/dendritic cells (DC) are shown. Data represent the combination of one (week 2) or two independent experiments (week 5 and 12) (n = 4–6). Dotted lines denote the mean numbers obtained from naive, uninfected WT mice. Statistical significance between groups is represented in the figure by *(0·01 < P < 0·05), **(0·005 < P < 0·01), ***(P < 0·005) and was evaluated by Student's t-test.

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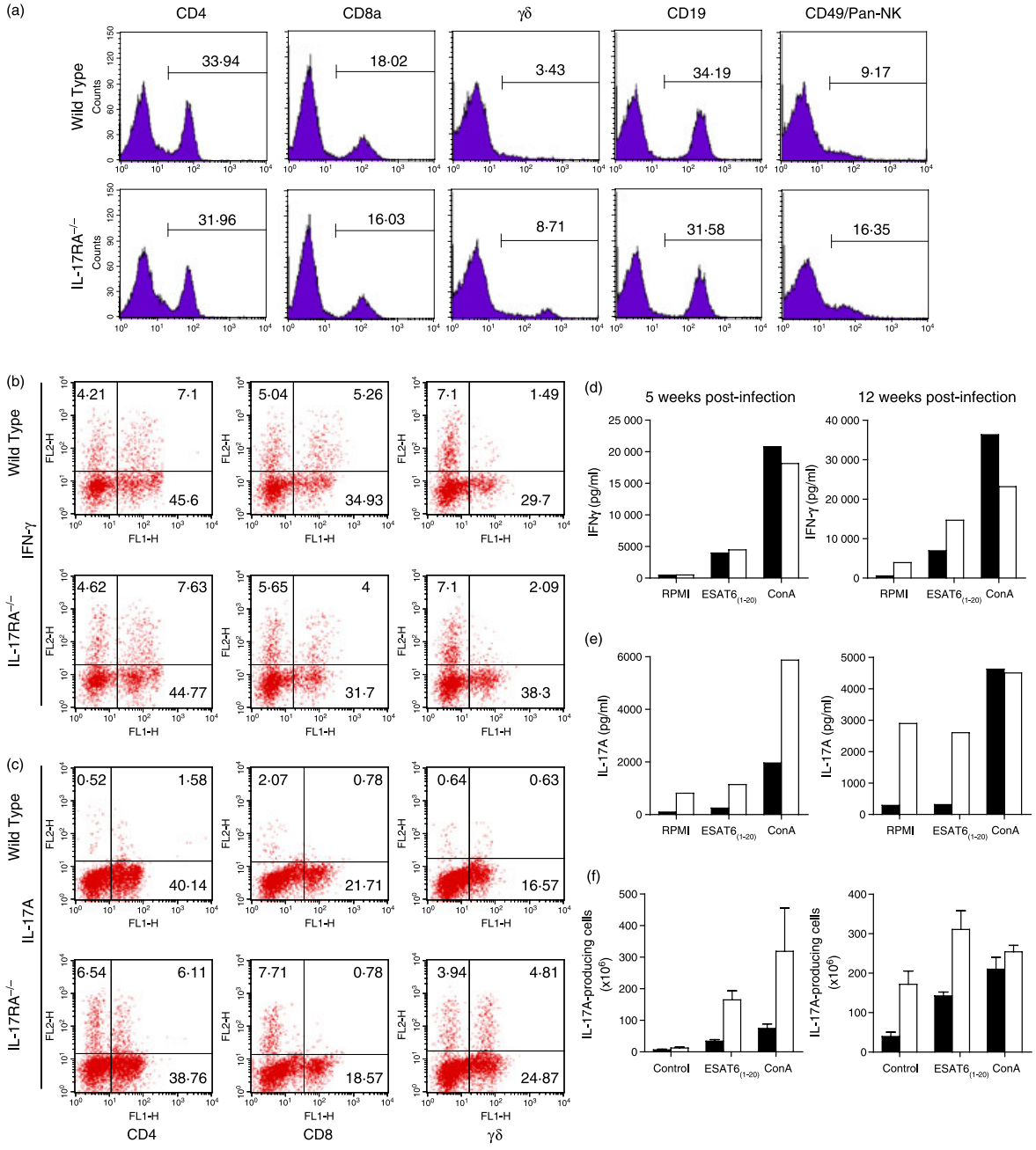

Figure 3. (a) CD4, CD8a, $\gamma\delta$, CD19 and CD49/Pan-NK staining of lung cells. Lung cells from wild-type (WT) or interleukin-17 receptor subunit A-deficient (IL-17RA$^{-/-}$) mice infected intratracheally with 10^3 colony-forming units (CFU) of *Mycobacterium tuberculosis* were harvested (n = 3) and pooled 5 weeks post-infection. The percentages of positive cells in the lymphocyte gate are expressed in each quadrant. The lymphocytes were gated by forward and side scatter plots. Data are representative of two independent experiments (see also Supplementary material, Fig. S2). Intracellular (b) interferon-γ (IFN-γ) or (c) IL-17A staining of CD4, CD8a and $\gamma\delta$ T cells. Lung cells from WT or IL-17RA$^{-/-}$ mice infected with 10^3 CFU of *M. tuberculosis* for 5 weeks were pooled (n = 3) and stimulated with PMA and ionomycin overnight. The percentage of positive cells in the lymphocyte gate is expressed in each quadrant. The lymphocytes were gated by forward and side scatter plot. (d) IFN-γ and (e) IL-17A production (pg/ml) by pulmonary cells of WT C57BL/6 (black bars) and IL-17RA$^{-/-}$ (white bars) mice 5 or 12 weeks after infection. IFN-γ and IL-17A (pg/ml) in 72-hr culture supernatant of pooled lung cells (n = 5) stimulated with medium only (control), ESAT6$_{(1-20)}$ or polyclonal T-cell mitogen concanavalin A (Con A) was measured by ELISA. Data are representative of three independent experiments (see also Supplementary material, Fig. S3). (f) Number of IL-17A-producing lung cells (n = 5) was measured by ELISPOT 5 and 12 weeks after infection from WT (black bars) and IL-17RA$^{-/-}$ (white bars) mice. Lung cells were stimulated with medium only (control), ESAT6$_{(1-20)}$ or polyclonal T-cell mitogen Con A. Bars represent the mean values obtained for samples tested in triplicate ± SD. Data are representative of two independent experiments.

IL-17RA$^{-/-}$ mice, with CD4$^+$ and CD8$^+$ T cells being the main contributors while $\gamma\delta$ T cells produced little IFN-γ at 5 weeks after infection (Fig. 3b). In contrast, the IL-17A response was more important in IL-17RA$^{-/-}$ than in WT mice and cells producing IL-17A were CD4$^+$ and $\gamma\delta$ T cells. Less than 1% of CD8$^+$ T cells from WT or IL-17RA$^{-/-}$ mice produced IL-17A (Fig. 3c).

Mycobacteria-specific IFN-γ and IL-17A responses were measured in lung cell cultures stimulated with immunodominant I-A^b restricted ESAT6$_{(1–20)}$ peptide after 5 and 12 weeks of infection. Production of IFN-γ was comparable at 5 weeks post-infection in the two groups (confirming the intracellular cytokine staining results). However, at 12 weeks post-infection, mycobacteria-specific IFN-γ responses were about twofold higher in IL-17RA$^{-/-}$ than in WT mice (Fig. 3d and Supplementary material Figure S3). The IL-17A levels were at least fivefold higher in IL-17RA$^{-/-}$ than in WT mice at 5 and 12 weeks post-infection. Moreover, IL-17A levels were also higher in non-stimulated pulmonary cell cultures of IL-17RA$^{-/-}$ than in WT mice (Fig. 3e and Supplementary material Figure S3).

Increased numbers of IL-17A-producing cells were detected in IL-17RA$^{-/-}$ mice by ELISPOT, following *in vitro* stimulation with ESAT-6$_{(1–20)}$ peptide and the polyclonal mitogen Con A (week 5 only) (Fig. 3f). Confirming the ELISA results, the number of IL-17A spot-forming cells at week 12 was also strongly increased in IL-17RA$^{-/-}$ mice in the absence of antigenic or mitogenic stimulation.

Compromised cytokine response in lungs of IL-17RA$^{-/-}$ mice

To find out whether cytokines and chemokines other than IL-17A were affected in IL-17RA$^{-/-}$ mice, we measured the levels of IL-6, IL-1β, KC (CXCL1), IP-10 (CXCL10), IL-10, TNF-α and IL-13 in non-stimulated pulmonary culture supernatant of naive (uninfected) and infected WT and IL-17RA$^{-/-}$ mice. Levels of TNF-α were about twofold lower in IL-17RA$^{-/-}$ mice after 5 weeks of infection but were similar in both groups after 12 weeks. Levels of IL-1β and IP-10 (CXCL10) were twofold to threefold higher in IL-17RA$^{-/-}$ mice at 12 weeks post-infection, whereas no IL-13 was detected at any time-point tested (below detection: < 9·3 pg/ml, data not shown). Levels of the KC (CXCL1) and IL-6 were lower in both naive and infected IL-17RA$^{-/-}$ mice and also IL-10 levels were lower (sixfold) in IL-17RA$^{-/-}$ mice at week 12 and even below detection level (< 5·4 pg/ml) at week 5 and in naive IL-17RA$^{-/-}$ mice (Table 2).

Gene expression profiling in *M. tuberculosis* infected IL-17RA$^{-/-}$ and WT mice

Finally, to compare in more detail the inflammatory profile in the lungs of IL-17RA$^{-/-}$ and WT mice, we performed a gene expression profiling of pulmonary cells after 2, 5 and 12 weeks of infection. No significant differences were found in expression levels of *Tbet*, *RORγt*, *Foxp3* and *GATA3*, the respective transcription factors for Th1, Th17, regulatory T and Th2 CD4$^+$ T cells (Fig. 4). Expression level of *LIX* (*CXCL5*) was significantly lower in IL-17RA$^{-/-}$ mice than in WT mice at 5 weeks post-infection, which is consistent with the poor neutrophil infiltration observed in the lungs during the first weeks after infection as well as with the low levels of KC (CXCL1) detected in pulmonary cell supernatant. Expression of the *Lcn2* and *LRG47* was significantly lower in IL-17RA$^{-/-}$ mice at 5 weeks post-infection, whereas expression of *iNOS* was significantly higher in IL-17RA$^{-/-}$ mice at week 12 post-infection (Fig. 5).

Discussion

Here, we have revisited the role of IL-17A during *M. tuberculosis* infection using mice deficient in the IL-17 receptor A subunit (IL-17RA$^{-/-}$ mice) and we have shown that the IL-17RA pathway is essential for long-term control of this infection. Interleukin-17RA is necessary for

Table 2. Cytokine and chemokine levels (pg/ml) in lung cell culture supernatants of wild-type or interleukin-17 receptor subunit A-deficient (IL-17RA$^{-/-}$) mice before and 5 or 12 weeks after *Mycobacterium tuberculosis* infection

	Uninfected		5 weeks post-infection		12 weeks post-infection	
Lungs	Wild-type	IL-17RA$^{-/-}$	Wild-type	IL-17RA$^{-/-}$	Wild-type	IL-17RA$^{-/-}$
IL-6	211	< 2·2	5109	2301	6227	1508
IL-1β	< 34·3	< 34·3	223	364	298	1030
KC	1175	272	1133	602	2701	1284
IP-10	< 9·8	19	878	796	1595	2634
IL-10	104	< 5·4	434	< 5·4	631	100
TNF-α	10	12	212	105	396	403

IL-6, interleukin-6; IP-10, CXCL10; KC, CXCL1; TNF-α, tumour necrosis factor-α.

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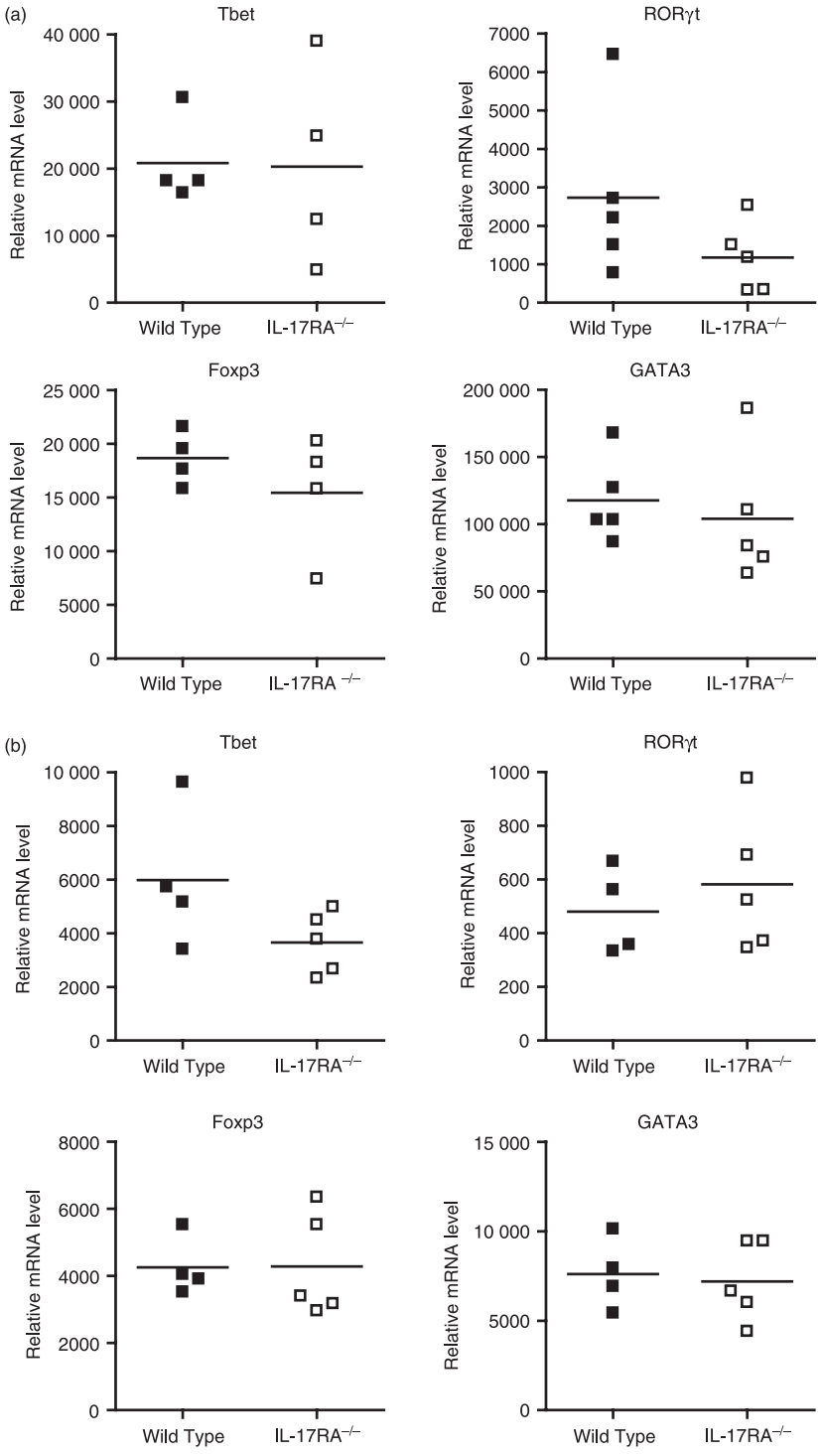

Figure 4. Messenger RNA expression of *Tbet*, *RORγt*, *Foxp3* and *GATA3* by pulmonary cells of C57BL/6 wild-type (■) and interleukin-17 receptor subunit A-deficient (IL-17RA$^{-/-}$) mice (□), (a) 2, (b) 5 and (c) 12 weeks after intratracheal instillation of *Mycobacterium tuberculosis* (10^3 colony-forming units). Data are expressed as relative mRNA levels, normalized against reference housekeeping gene (HMBS; hydroxymethylbilane synthase).

signal transduction mediated by IL-17A, IL-17F and IL-17E (IL-25).[24,25] In our study we could only detect IL-17A, but not IL-17F or IL-17E, after *M. tuberculosis* infection (data not shown) and therefore we assumed that using IL-17RA$^{-/-}$ mice was a valid approach for the study of IL-17A signalling in *M. tuberculosis* infection.

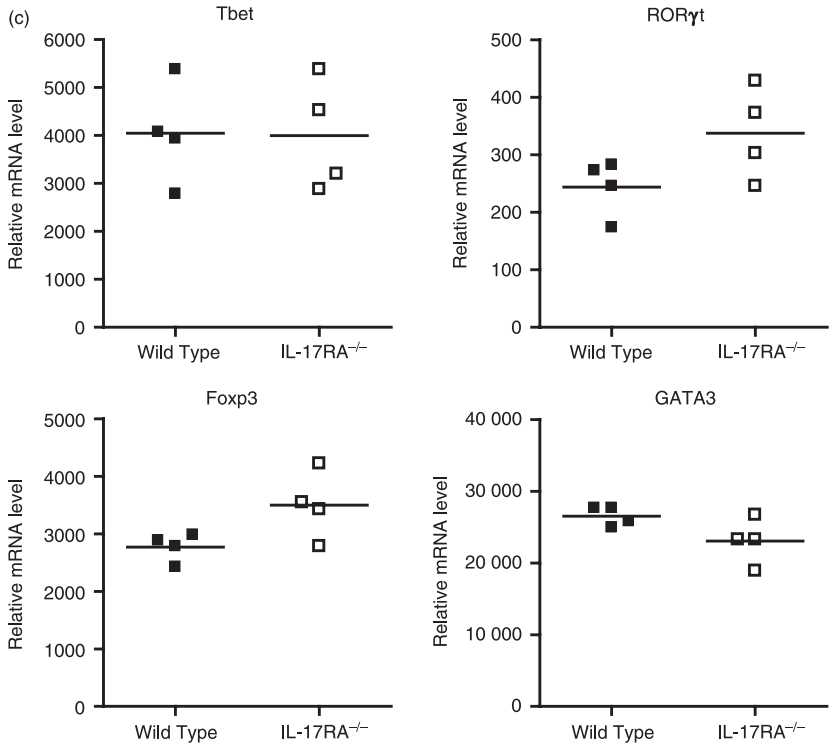

Figure 4. *(Contiuned.)*

In the absence of IL-17RA signalling, a dramatic increase of IL-17A production by CD4$^+$ T cells and $\gamma\delta$ T cells (but not by CD8$^+$ T cells) was observed by intracellular cytokine staining, ELISA and ELISPOT. Moreover, $\gamma\delta$ and natural killer cell number in the lungs was also higher in IL-17RA$^{-/-}$ mice after infection and the latter cell subset might also contribute to the increased IL-17A production, as reported in the context of toxoplasmosis infection.[26] Smith *et al.*[27] have reported that IL-17A can inhibit the expansion of IL-17A-producing T cells through a feed-back inhibition via IL-17 receptor and actually, the overproduction of IL-17A is part of the IL-17RA$^{-/-}$ phenotype.[18]

Whereas IL-17A exerts its protective role against extracellular bacteria by efficient neutrophil recruitment, its role in defence against intracellular bacterial infection is more complex and may be influenced by bacterial dose and the route of infection.[28] Here we have shown that a defective IL-17RA-signalling pathway increases the susceptibility to both low-dose (10^3 CFU) intratracheal and high-dose (2×10^5 CFU) intravenous infection with *M. tuberculosis*, but that this increased susceptibility is only apparent after several months of infection. On the other hand, Khader *et al.*[13] and Aujla *et al.*[29] have reported that IL-17RA$^{-/-}$ mice were not more susceptible than WT mice following a low-dose (100 CFU) aerosol infection. As we have used a 10-fold higher infectious dose, this could indicate that activation of the IL-17RA pathway may be critical only when bacterial burden is high. This dose-dependency of susceptibility was also observed in IL-6$^{-/-}$ mice, which control a low dose, but succumb to a high infectious dose.[30,31]

Activation of the IL-17RA pathway mediates neutrophil recruitment to the lungs via the induction of chemokines such as KC (CXCL1) and LIX (CXCL5). This was confirmed by our observation that IL-17RA$^{-/-}$ mice were defective in neutrophil recruitment during at least the first 5 weeks of infection and also defective in early production of these two chemokines. The role of neutrophils in protective immunity against *M. tuberculosis* remains unclear and while one report demonstrated an increased bacterial burden when neutrophils were depleted, another report failed to detect any difference.[32,33] In mice vaccinated intradermally with BCG, the depletion of the early neutrophil influx induces a population of CD11b$^+$-Ly-C^{int} Ly-6G$^-$ myeloid-derived suppressor cells that impair T-cell priming in the draining lymph node.[34] Likewise, Blomgran *et al.*[35,36] demonstrated that neutrophils are necessary for the timely initiation of the adaptive immune response by supporting effective migration of infected DCs harbouring *M. tuberculosis* to the lung-draining lymph node and contributing to activation of antigen-specific CD4$^+$ T-cell responses. This is in line with our findings on the low lymphocyte infiltration in BAL fluid that was observed in IL-17RA$^{-/-}$ mice.

Likewise in humans, some reports have indicated the importance of neutrophils in immune defence against tuberculosis. Hence, the risk of TB infection among

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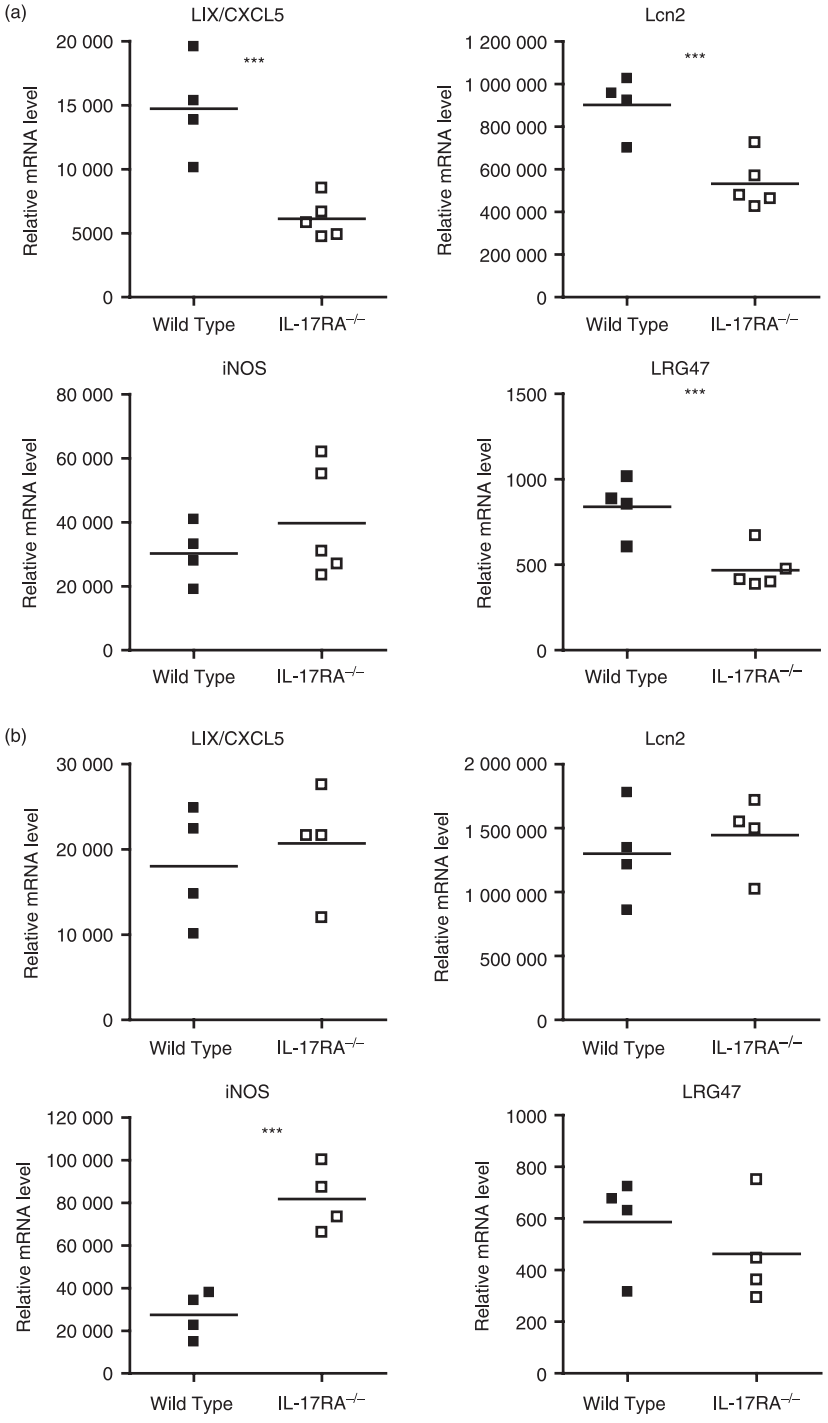

Figure 5. Messenger RNA expression of *LIX* (*CXCL5*), *Lcn2*, *iNOS* and *LRG47* by pulmonary cells of wild-type (WT) (■) and interleukin-17 receptor subunit A-deficient (IL-17RA$^{-/-}$) mice (□), (a) 5 and (b) 12 weeks after intratracheal instillation of *M. tuberculosis* (10^3 CFU). Data are expressed as relative mRNA levels, normalized against reference housekeeping gene (HMBS; hydroxymethylbilane synthase) and are individual for each mouse. Statistical significance between groups is represented in the figure by *($0{\cdot}01 < P < 0{\cdot}05$), **($0{\cdot}005 < P < 0{\cdot}01$), ***($P < 0{\cdot}005$) and was evaluated by Student's *t*-test.

household contacts of patients with TB is inversely related to peripheral blood neutrophil count, and killing of *M. bovis* BCG in an *in vitro* human whole-blood assay is significantly impaired by neutrophil depletion.[37] Moreover, humans exhibit a transcriptional signature in peripheral blood that indicates a role of neutrophils in response to active pulmonary TB.[38] As treatment of autoimmune diseases with anti-IL-17A antibodies is currently

being tested in clinical studies,[39] a careful monitoring of patients with respect to possible reactivation of TB, as described for anti-TNF-α treatment, may be warranted.

Both IFN-γ and TNF-α play a determining role in the outcome of *M. tuberculosis* infection.[4,28,40] Although IL-17RA$^{-/-}$ mice were more susceptible to *M. tuberculosis* than WT mice, their mycobacteria-specific IFN-γ response was not compromised and at week 12 post-infection levels of IFN-γ and of the microbicidal enzyme iNOS levels were even higher than in WT mice. In recent years, it has become increasingly clear that high levels of IFN-γ do not necessarily correlate with increased protection.[16,41] Notably, the expression of LRG47, another anti-mycobacterial agent dependent on IFN-γ,[42] was also comparable in both groups.

Tumour necrosis factor-α, IL-6, IL-1β and IL-10 are innate cytokines modulating the immune response against *M. tuberculosis*[28] and TNF-α is essential for long-term maintenance of the granuloma and the control of bacterial growth.[43–45] TNF-α and two other pro-inflammatory cytokines, IL-6 and IL-1β, are induced by IL-17A whereas IL-6 and IL-1β are also important for the maintenance of IL-17A-producing cells.[46] Moreover, IL-17A can act in synergy with TNF-α and IL-1β to amplify their effect.[47] As expected, IL-6 levels were decreased in IL-17RA$^{-/-}$ mice at both 5 and 12 weeks post-infection, whereas TNF-α levels were also decreased (but only at week 5) and IL-1β levels were even increased at both time-points. Interestingly, levels of the regulatory cytokine IL-10, which suppresses macrophage and DC function, limits recruitment of Th1 cells to the lungs of infected mice and limits pathogen clearance during early immune responses to *M. tuberculosis*,[48] were very low in IL-17RA$^{-/-}$ mice and even below detection levels at the early stages. Neutrophils secrete IL-10 after infection with *M. tuberculosis*, and neutrophil depletion leads to reduced IL-10 production in the lungs.[49] It is possible that the low IL-10 levels observed in IL-17RA$^{-/-}$ mice are directly related to the low neutrophil numbers initially recruited to the lungs. Higgins *et al.*[50] have shown that the absence of IL-10 leads to *M. tuberculosis* regrowth during chronic infection and results in the premature death of the mice. Hence, it is possible that low IL-10 production is responsible for the defective long-term control of *M. tuberculosis* in IL-17RA$^{-/-}$ mice, but more work is needed to verify this hypothesis.

Interleukin-17RA-signalling might also have a direct effect on the antimicrobial properties of infected macrophages, as has been suggested for *Bordetella pertussis*[51] and *Francisella tularensis*.[52] However, stimulation of infected bone-marrow-derived macrophages with recombinant IL-17A had no direct effect on *M. tuberculosis* growth, in contrast to treatment with IFN-γ, which resulted in decreased bacterial growth in bone-marrow-derived macrophages from both WT and IL-17RA$^{-/-}$ mice, suggesting that macrophages from IL-17RA$^{-/-}$ mice can respond equally well to IFN-γ as macrophages from WT mice (data not shown).

In conclusion, we hypothesize that the increased susceptibility to *M. tuberculosis* of IL-17RA$^{-/-}$ mice is related to a delay in neutrophil recruitment to the lungs during the first weeks of the infection, associated with an aberrant cytokine response (decreased IL-6 and IL-10 and increased IL-1β), rather than to a defective cognate mycobacteria-specific Th1 response. Our findings underscore the important role of innate immune defence mechanisms for the protection against the intracellular pathogen *M. tuberculosis* and the complexity of the cytokine network.

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Disclosures

The authors have no conflict of interest to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Wild-type (WT) C57BL/6 (■, n = 10–11) and interleukin-17 receptor subunit A-deficient (IL-17RA$^{-/-}$) mice (□, n = 10–11) were infected (A) intratracheally with 5×10^3 colony-forming units (CFU) or (B) intravenously with 2×10^5 CFU of *Mycobacterium tuberculosis*.

Figure S2. CD4, CD8a, γδ, CD19 and CD49/Pan-NK staining of lung cells (results of experiment independent of that shown in Fig. 3a).

Figure S3. Interferon-γ (IFN-γ) and interleukin-17 (IL-17A) production (pg/ml) by pulmonary cells of wild-type (WT) C57BL/6 (■) and IL-17 receptor subunit A-deficient (IL-17RA$^{-/-}$) (□) mice 5 weeks (A) or 12 weeks (B) after infection.